Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12378–12382, December 1994
Cell Biology

Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction

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Communicated by Melvin Calvin, August 26, 1994 (received for review June 23, 1994)

ABSTRACT **Extracellular matrix (ECM) profoundly influences the growth and differentiation of the mammary gland epithelium, both in culture and *in vivo*. Utilizing a clonal population of mouse mammary epithelial cells that absolutely requires an exogenous ECM for function, we developed a rapid assay to study signal transduction by ECM. Two components of the cellular response to a basement membrane overlay that result in the expression of the milk protein β-casein were defined. The first component of this response involves a rounding and clustering of the cells that can be physically mimicked by plating the cells on a nonadhesive substratum. The second component is biochemical in nature, and it is associated with β_1 integrin clustering and increased tyrosine phosphorylation. The second component is initiated in a morphology-independent manner, but the proper translation of this biochemical signal into a functional response requires cell rounding and cell clustering. Thus, physical and biochemical signal transduction events contribute to the ECM-dependent regulation of tissue-specific gene expression in mouse mammary epithelial cells.**

Cell structure and function are profoundly influenced by the extracellular matrix (ECM), both during development and in the adult organism (1). This is particularly evident in the mouse mammary gland, where modifications of the ECM at the stromal–epithelial interface influence ductal branching, end-bud development, and epithelial proliferation in the prepubertal gland (2–4). During pregnancy and lactation an intact basement membrane is required for function, and the remodeling of this specialized ECM contributes to involution after weaning (4–6). Additionally, there is overwhelming evidence that the ECM strongly influences mammary epithelial cell differentiation in culture (7–9). When placed upon a reconstituted basement membrane gel, these cells undergo morphogenesis to form spheroids that exhibit many of the structural and functional characteristics of alveoli *in vivo* (10, 11).

From a functional standpoint, an important aspect of mammary epithelial cell differentiation is the expression of the milk protein genes. This is controlled by both soluble lactogenic hormones and the ECM (12). Specifically, expression of the abundant milk protein β-casein is regulated by prolactin (PRL; ref. 13) and basement membrane. The latter may be endogenously produced (9, 14) or exogenously added (15). Basement membrane-dependent β-casein expression requires functional β_1-integrins (15), and it is regulated transcriptionally by an ECM response element located upstream of the gene (16, 17). Thus, interactions between cells and the ECM generate signals at the cell surface that are ultimately transmitted to the nucleus. While recent studies have begun to unravel the PRL signal transduction pathway (18, 19), the signals generated by cell–ECM interactions in this system have not yet been identified.

Utilizing a homogeneous mammary epithelial cell clone that cannot synthesize its own basement membrane, and thus absolutely requires the addition of an exogenous ECM for β-casein expression (20), we have kinetically separated the cellular response to ECM from that to PRL. Physical interactions with the ECM, which was added to cell monolayers in the form of a pliable basement membrane overlay (ref. 21; C. H. Streuli and M.J.B., unpublished data), resulted in a cell rounding and cell clustering that was necessary but not sufficient for β-casein expression. A biochemical signal that was associated with β_1 integrin clustering and increased tyrosine phosphorylation was further required.

METHODS AND MATERIALS

Homogeneous scp2 mouse mammary epithelial cells (20) were isolated from the heterogeneous CID 9 cell strain (16) by limiting dilution cloning. Cells were plated at a density of 5×10^4 cells per cm^2 in DMEM (Dulbecco's modified Eagle's medium)/F12 medium supplemented with 2% fetal bovine serum, insulin (5 μg/ml), and hydrocortisone (1 μg/ml) for 24 h. All further treatments were carried out in serum-free medium. Cells were plated as a monolayer on tissue culture plastic, or as rounded cell clusters on the nonadhesive substratum poly(2-hydroxyethyl methacrylate) (polyHEMA, Sigma). PolyHEMA-coated plates were prepared as described (22), using an initial concentration of 0.25 mg/ml in 95% ethanol before drying. Ovine PRL (3 μg/ml, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda) was added at the time indicated in each experiment. Engelbreth–Holm–Swarm tumor basement membrane ECM was prepared as described (23). It was then diluted to 1% (vol/vol, approximately 200 μg of total protein per ml) in DMEM/F12 and added to the cells as an ECM overlay (21). The phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 100 ng/ml) and genistein (5 μM, Life Technologies, Grand Island, NY) were added for the first 3 days of the 5-day ECM overlay pretreatment period, as this ensured that their effects, both morphological and functional, were reversible.

Immunoprecipitation of milk protein from [^{35}S]methionine pulse-labeled cell extracts was carried out as described (9). In the ECM overlay assay, scp2 cells synthesize a 30-kDa β-casein doublet, a 43-kDa α-casein doublet, and a relatively broad protein band of approximately 70 kDa that has been tentatively identified as either lactoferrin (Lf) or transferrin

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Abbreviations: ECM, extracellular matrix; LN, laminin; PRL, prolactin; polyHEMA, poly (2-hydroxyethyl methacrylate); DAPI, 4′,6-diamidino-2-phenylindole; TPA, 12-*O*-tetradecanoylphorbol 13-acetate (phorbol 12-myristate 13-acetate); Lf, lactoferrin; Tf, transferrin.

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(Tf) (20). For Northern blotting, RNA was extracted with guanidinium isothiocyanate (24), electrophoresed through 1% agarose gels under denaturing conditions, and transferred to nylon membranes. Membranes were then hybridized with a ^{32}P-labeled β-casein probe (courtesy J. Rosen, Baylor College of Medicine, Houston). For immunoblotting, whole cell lysates (100 μg of total protein) containing 1 mM orthovanadate were separated by SDS/8% PAGE, transferred to Immobilon membranes (Millipore), and probed with an antiphosphotyrosine polyclonal antibody (Transduction Labs, Lexington, KY). Antibody binding was visualized with enhanced chemiluminescence (Amersham). In antiphosphotyrosine experiments, laminin (LN, 50 μg/ml; Sigma) purified from the Englebreth–Holm–Swarm tumor was used for the ECM overlay. This laminin preparation contains entactin but no growth factors and efficiently induces β-casein expression (21). Its use here ensured that the changes in tyrosine phosphorylation observed were not due to contaminating soluble growth factors present in the basement membrane preparation. For immunofluorescence, paraformaldehyde-fixed, Triton X-100-permeabilized cultures were treated with a rabbit polyclonal antibody against β_1 integrin (Chemicon) or a mouse monoclonal antibody raised against rat β-casein (courtesy of C. Kaetzel, University of Kentucky College of Medicine, Lexington, KY). Antibody binding was visualized indirectly with a species-specific Texas red-labeled biotin–streptavidin complex. Filamentous actin was visualized with rhodamine-phalloidin (Molecular Probes) and nuclei were fluorescently stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma).

RESULTS

ECM Overlay-Dependent β-Casein Expression. The clonal mouse mammary epithelial cell line scp2 forms flattened epithelial monolayers in two-dimensional culture. Unlike other functional but heterogeneous mammary lines such as HC11 (25) and CID 9 (16), homogeneous scp2 cells absolutely require an exogenously added ECM to express β-casein, even at high cell density (20).

A 1% solution of reconstituted basement membrane ECM was overlaid upon scp2 monolayers for 7 days in the continuous presence of PRL (Fig. 1). Despite the fact that the ECM formed a flocculent precipitate on top of the cells that was clearly visible after 1 day (Fig. 1*A*), β-casein expression did not begin until day 3, and it increased gradually thereafter (Fig. 1 *B* and *C*). Synthesis of other milk proteins also increased after 3–5 days, albeit with slightly different kinetics. This delayed onset in milk protein gene expression has been described previously by using primary cultures plated on (11), or within (15), reconstituted basement membrane gels.

Utilizing the overlay technique, we were able to directly observe the morphological response to ECM addition. After 1 day the cells had retracted slightly from the underlying substratum, and by day 3 they were clearly rounded. These rounded cells then came together to form cohesive clusters without central lumina at day 5. Therefore, profound ECM-dependent changes in morphology took place prior to β-casein expression.

Cell Rounding and Cell Clustering Accelerate ECM-Dependent β-Casein Expression. To determine the role that these changes in morphology play in β-casein expression, scp2 cells were plated as monolayers on tissue culture plastic or they were preclustered on the nonadhesive substratum polyHEMA for 5 days (Fig. 2). A subsequent addition of PRL alone did not induce β-casein expression in either plating condition (not shown, and see below). In contrast, when ECM and PRL were added to the cultures together, β-casein expression occurred within 8 h in cells plated on polyHEMA

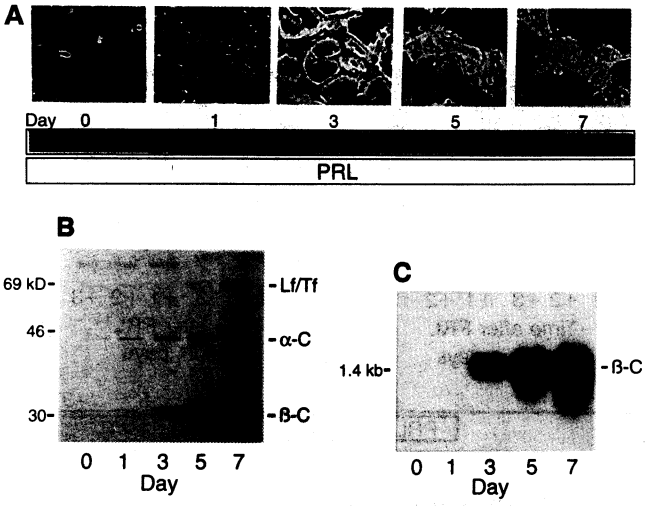

FIG. 1. PRL and an ECM overlay together induce β-casein expression. scp2 mouse mammary epithelial cell monolayers were treated with PRL and an ECM overlay continuously for 7 days. (*A*) The morphological response to the PRL/ECM treatment was analyzed. The cells began to retract from the substratum after 1 day, became rounded at 3 days, and formed cohesive clusters by day 5. The flocculent material visible atop the cells is the ECM overlay. (Phase-contrast microscopy; ×75.) (*B*) Cells were pulse-labeled with [^{35}S]methionine on the days indicated. Cell lysates were then analyzed for milk protein synthesis by immunoprecipitation. The 30-kDa β-casein doublet (β-C) was first synthesized on day 3. α-Caseins (α-C) and a 70-kDa band that consisted of Lf or Tf (20) were also synthesized. (*C*) RNA was extracted on the days indicated and analyzed by Northern blotting. The 1.4-kb β-casein mRNA (β-C) was first detected after 3 days.

(Fig. 2). Therefore, rounding and cell clustering greatly accelerated the response to ECM.

Cell Rounding Is Necessary but Not Sufficient for β-Casein Expression. When scp2 cells were plated as monolayers on tissue culture plastic for 5 days, they remained flat, which was reflected by their large nuclear profiles (Fig. 3). In contrast, rounded cells clustered on polyHEMA contained nuclei that were small and refractile. A subsequent 3-day treatment with PRL alone did not induce β-casein protein

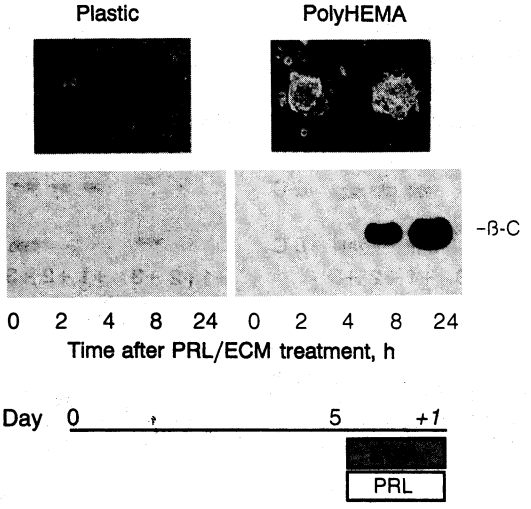

FIG. 2. Cell rounding and cell clustering accelerate ECM-dependent β-casein expression. Cells were plated as monolayers on tissue culture plastic, or as clusters on the nonadhesive substratum, polyHEMA, and maintained in serum-free medium for 5 days. Cultures were then treated simultaneously with PRL and ECM for 24 h. β-Casein (β-C) expression was assayed by Northern blotting analysis throughout this treatment period.

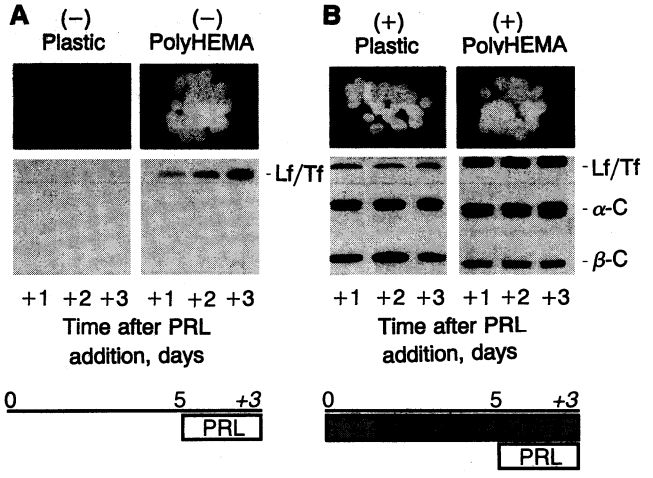

FIG. 3. ECM pretreatment leads to rapid and maximal β-casein synthesis. Cells were plated as monolayers on tissue culture plastic or as clusters on polyHEMA. They were maintained in serum-free medium (*A*, −) or were pretreated with ECM (*B*, +) for 5 days. Nuclear morphology was then assessed by DAPI staining. (Fluorescence microscopy; ×170.) PRL was then added for an additional 3 days. Milk protein synthesis was analyzed by immunoprecipitation at 24-h intervals after PRL addition.

synthesis under either of these plating conditions (Fig. 3*A*). It is interesting to note, however, that the synthesis of a 70-kDa milk protein (Lf or Tf) did occur in the cells clustered on polyHEMA.

When flat monolayers cultured on tissue culture plastic were pretreated with an ECM overlay for 5 days, clusters formed, cell rounding occurred, and the nuclei became small and refractile (Fig. 3*B*). Addition of PRL after this ECM pretreatment induced maximal synthesis of β-casein and the other milk proteins after only 1 day. This same rapid and maximal milk protein synthesis also occurred when cells clustered on polyHEMA were pretreated with ECM. These data have two important implications: first, the cellular responses to ECM and PRL can be separated kinetically, and, second, cell rounding and cell clustering alone, which lead to changes in nuclear morphology, are not sufficient for β-casein expression.

The phorbol ester TPA inhibits β-casein expression in primary mammary epithelial cells (26). It also prevented ECM-mediated cell rounding and clustering. As a result, when flat scp2 cells on tissue culture plastic were pretreated with both TPA and ECM, they remained flat with large nuclear profiles, and the rapid synthesis of β-casein and other milk proteins was severely curtailed (Fig. 4*A*). However, when scp2 cells were clustered on polyHEMA prior to TPA and ECM pretreatment, they remained rounded with small nuclear profiles, and the inhibition of milk protein synthesis was abrogated. Therefore, TPA prevented milk protein synthesis in a morphology-dependent manner.

Given the fact that changes in cell morphology appear to be necessary but not sufficient for β-casein synthesis, we postulated that additional ECM-mediated signal(s) might also be required. Thus, various kinase inhibitors were added to cultures during ECM pretreatment in an effort to distinguish which signal transduction pathways might be involved. Inhibitors of protein kinase A (H-89) and protein kinase C

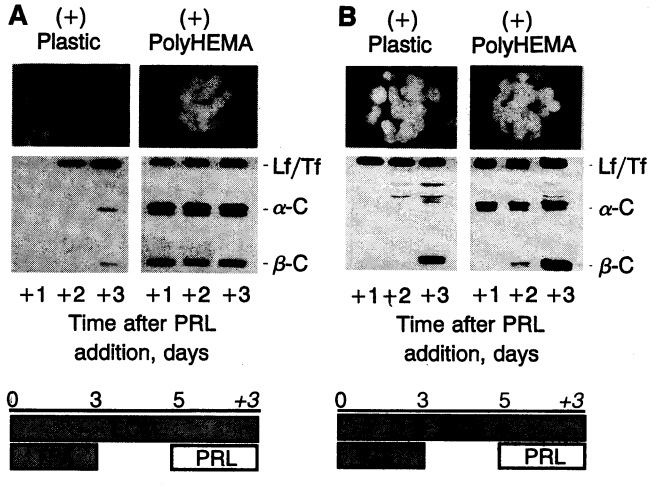

FIG. 4. TPA and genistein inhibit ECM-dependent β-casein synthesis. Cells plated on tissue culture plastic or polyHEMA were pretreated with ECM (+) in the presence of either TPA (*A*) or genistein (GS, *B*). TPA and genistein were added only during the first 3 days of ECM pretreatment to ensure that their effects were reversible. At the conclusion of pretreatment, nuclear morphology was assessed by DAPI staining. PRL was then added for an additional 3 days. Milk protein synthesis was analyzed by immunoprecipitation at 24-h intervals after PRL addition.

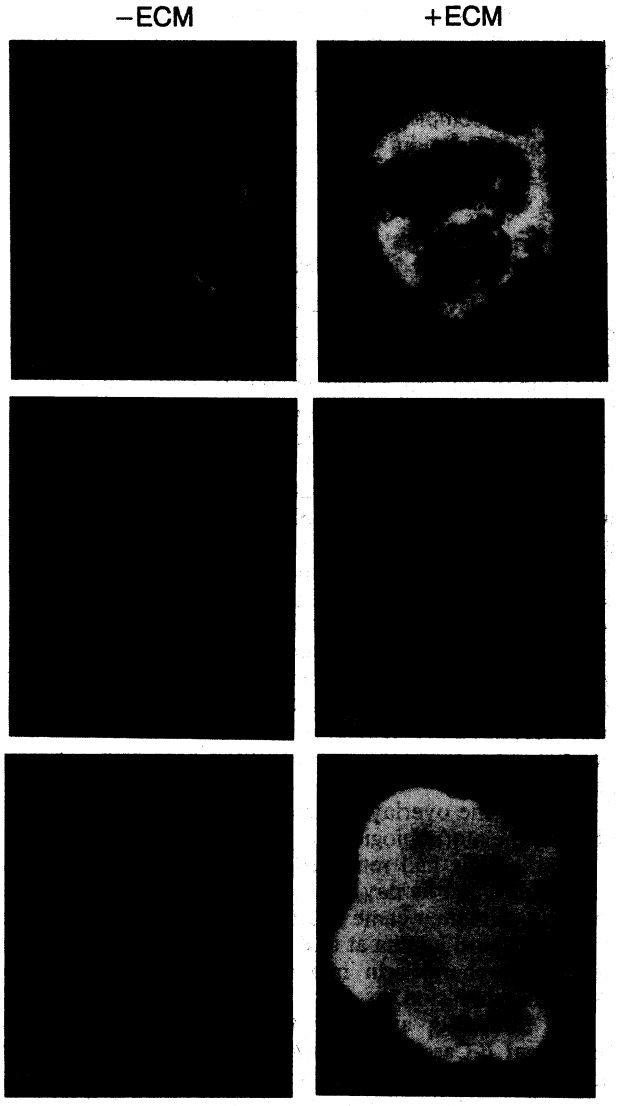

FIG. 5. ECM overlay induces β_1 integrin clustering in cells on polyHEMA. Cells plated as clusters on polyHEMA were maintained in serum-free medium without ECM (*A*, *C*, and *E*) or they were overlaid with ECM for 24 h (*B*, *D*, and *F*). The cells were then fixed and stained for β_1 integrin (*A* and *B*), filamentous actin (*C* and *D*), or β-casein (*E* and *F*). (Fluorescence microscopy; ×590.)

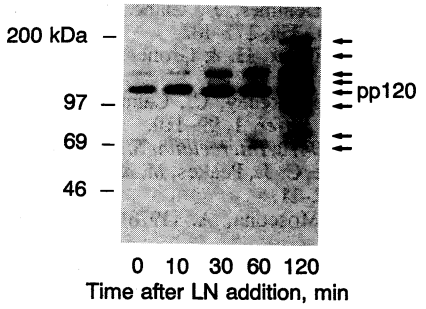

FIG. 6. An LN overlay induces increased tyrosine phosphorylation in cell clusters. Cells plated as clusters on polyHEMA were overlaid with LN (50 μg/ml) for 2 h. Phosphotyrosine-containing proteins were identified by Western blotting. Molecular mass markers are indicated on the left, while the arrows on the right identify proteins whose phosphate signal increases during LN treatment. One of the more prominent species is a 120-kDa phosphoprotein (pp120). LN was used as the overlay in this assay to ensure that the observed changes in phosphorylation were ECM specific.

(calphostin C) had no effect (not shown). In contrast, the tyrosine kinase inhibitor genistein inhibited rapid β-casein synthesis under both the tissue culture plastic and polyHEMA culture conditions (Fig. 4*B*). Unlike TPA, genistein had no discernible effect on cell clustering, nuclear size, or synthesis of the 70-kDa Lf or Tf protein. Therefore, the perturbation of one or more tyrosine kinases appeared to specifically inhibit β-casein synthesis in a morphology-independent manner.

ECM Overlay Increases β_1 Integrin Clustering and Tyrosine Phosphorylation in Cell Clusters. What is the nature of the signal induced by ECM after the cells have been rounded and clustered? β-Casein expression in mammary epithelial cells requires functional β_1 integrin receptors (15), and the clustering of such receptors precedes biochemical signaling in other systems (27). Therefore, β_1 integrin localization was examined in scp2 cells cultured on polyHEMA before and after ECM addition. While there is slight receptor clustering in the absence of ECM (Fig. 5*A*), it is apparent that the quantity and size of the clusters were increased in the presence of ECM (Fig. 5*B*). This clustering was also associated with ECM-mediated increases in tyrosine phosphorylation. Over a 2-h ECM treatment period, phosphorylation was increased in at least eight protein species (Fig. 6). One of the more prominent bands was a 120-kDa phosphoprotein that has been observed in a number of other systems (27).

When cells were first clustered on polyHEMA, ECM addition did not affect cell shape, filamentous actin localization, or nuclear morphology (Fig. 5 *C* and *D* and Figs. 3 and 4). With or without ECM treatment, actin filaments were restricted to the cell cortex in these rounded cells. However, β-casein was expressed only in the ECM-treated cultures (Fig. 5 *E* and *F*). Thus, subtle changes in either the cytoskeleton or the nuclear skeleton may have taken place. Clustering of β_1 integrin receptors also occurred when cells on tissue culture plastic were treated with ECM, but in these flattened cells, actin filaments were dispersed much more diffusely throughout the cytoplasm (not shown). Therefore, both integrin clustering and the dramatic changes in the cytoskeleton that are associated with cell rounding appear to be necessary to initiate the functional signal transduction that leads to β-casein expression in these cells.

DISCUSSION

ECM–integrin complexes serve to physically link the ECM to the cytoskeleton (28, 29). As a result, adhesion to a rigid ECM leads to increased cytoskeletal resistance and cell spreading, while a malleable gel of the same ECM leads to decreased resistance and cell rounding (7, 10, 30). Such differences in cell shape have important functional consequences. In tissue culture, cell spreading is often a prerequisite for proliferation, while cell rounding is often associated with the maintenance of the differentiated state in epithelial cells (22, 31, 32). The mechanism(s) responsible for such fundamental alterations in cellular phenotype are not yet clear. However, there is evidence that ECM-mediated changes in cell shape lead to changes in the cytoskeleton and in the insoluble nuclear matrix that can have profound effects on gene activity (33, 34). Distinct from this physical signal transduction, ECM–integrin interactions also initiate a variety of biochemical signaling events. These include the induction of calcium transients, changes in cAMP levels, redistribution of PI-3 kinase (1-phosphatidylinositol 3-kinase), activation of the Na^+/H^+ antiporter, and increases in tyrosine phosphorylation (27, 35).

We had previously concluded that a single mammary epithelial cell in contact with a basement membrane gel is capable of synthesizing β-casein in the absence of cell–cell contact and polarity (15). This implied that signals emanating from the ECM alone were both necessary and sufficient for tissue-specific gene expression. Utilizing the ECM-dependent cell line scp2 together with a basement membrane overlay assay, we have now identified two components of this signaling pathway. The first component is physical, and it involves cell rounding and cell clustering. The second component is biochemical, and it involves increases in tyrosine phosphorylation that are associated with integrin clustering. Therefore, it is likely that single cells suspended in a basement membrane gel were able to express β-casein because they were already rounded (15). Indeed, when individual rounded cells are plated on the nonadhesive substratum polyHEMA and then overlaid with ECM, they too express β-casein, albeit slowly, over a number of days (C.D.R. and M.J.B., unpublished observations). In contrast, when these rounded cells come together to form cohesive clusters, β-casein expression is initiated very rapidly, within hours (Fig. 2). ECM-dependent cell clustering and this rapid β-casein expression could be prevented by phorbol ester treatment. Thus, as has been demonstrated in other systems (36–38), phorbol esters appear to inhibit mammary epithelial cell differentiation either by increasing adhesion to the underlying substratum or by disrupting the cytoskeleton such that cell rounding can no longer occur in response to ECM treatment. This was confirmed by preclustering scp2 cells on the nonadhesive substratum polyHEMA, which strongly abrogated TPA's ability to inhibit β-casein synthesis.

While cell rounding and cell clustering were necessary for rapid β-casein expression, they were not sufficient. Cell clusters expressed β-casein only after they were overlaid with ECM, which also increased β_1 integrin clustering and cytoplasmic tyrosine phosphorylation. Integrin clustering is a prerequisite for ECM-mediated increases in tyrosine phosphorylation (39). Functionally, signals of this nature have been previously linked to cell adhesion, spreading, and migration (40–42). Given the fact that genistein inhibited ECM-dependent β-casein expression, this biochemical signal also appears to be involved in the differentiation of mammary epithelial cells.

In this paper, we have focused on β-casein expression as a functional endpoint. However, complete functional differentiation of the mammary epithelial cell requires a much more demanding tissue organization than the simple cell clustering that has been described here. For example, ECM-dependent expression of whey acidic protein (WAP) occurs only after complete alveolar histogenesis has taken place (43, 44). Therefore, as structural and functional differentiation proceeds, ECM-mediated signaling must increase in com-

plexity. With many of the critical control points for milk protein production now in hand, the task ahead will be to determine precisely how these ECM-mediated physical and biochemical signals are integrated to initiate a series of tissue-specific responses at the level of gene expression.

We thank Nina Bailey for her expert technical assistance. This work was supported by a grant from the Health Effects Research Division, Department of Energy (Contract DE-AC03-76SF00098). C.D.R. is a National Cancer Institute of Canada postdoctoral fellow. P.Y.D. was supported by a fellowship from the Ligue Nationale Française contre le Cancer and the Association Claude Bernard.

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